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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

Treatment of tissue and cells.

by K. Lang and G. Siebert.

Hoppe-Seyler/Thierfelder, Handbuch der physiologisch- und pathologisch-chemischen analyse, 10th ed. vol. II, part II, p. 567-571 & 573 (1955).

Isolation in aqueous media.

Production of cell nuclei in hypertonic sucrose solution after Lang and Siebert (1). The bleeding of organs of small animals is conducted as described on p. 562. Larger organs, e.g. placentae or swine kidneys are best treated via a vascular trunk; a 10% sucrose solution is injected by means of a glass syringe; numerous cuts in the organ's surface allow the solution to escape; this method also permits very thorough bleeding.

The weighed tissue is subjected to preliminary diminution with scissors, knife, meat grinder or pressing through sieves, preferably into particles not larger than 1-3 mm on a side. The choice of method depends primarily on the tissual consistency and the purpose of subsequent tests; pressing through a plastic sieve, for instance, may be accomplished without any contact of the tissue with metal parts. Next, the tissue is further reduced in size in a modified glass homogenizer, which is described in detail below, with addition of 1.5 vol. 30% sucrose solution. It is subsequently forced through a filter cloth (2), e.g. with a porcelain pestle, and may now be used for insertion in the cell nucleus grinder.

The cell nucleus grinder (3) (Fig. 9) consists essentially of a piston of chromed V2A steel, which rotates around its axis in a cylinder of the same material; both parts have polished surfaces and are 1° conical; the gap between them amounts to ca. 0.1 mm. An adjustment allows the lowering and raising of the piston while the machine is in action, so that the width of the gap may be adapted to the requirements of the ground tissue. Turbulence does not occur under the operating conditions of the cell nucleus grinder (cf. also Vol. II, p. 101).

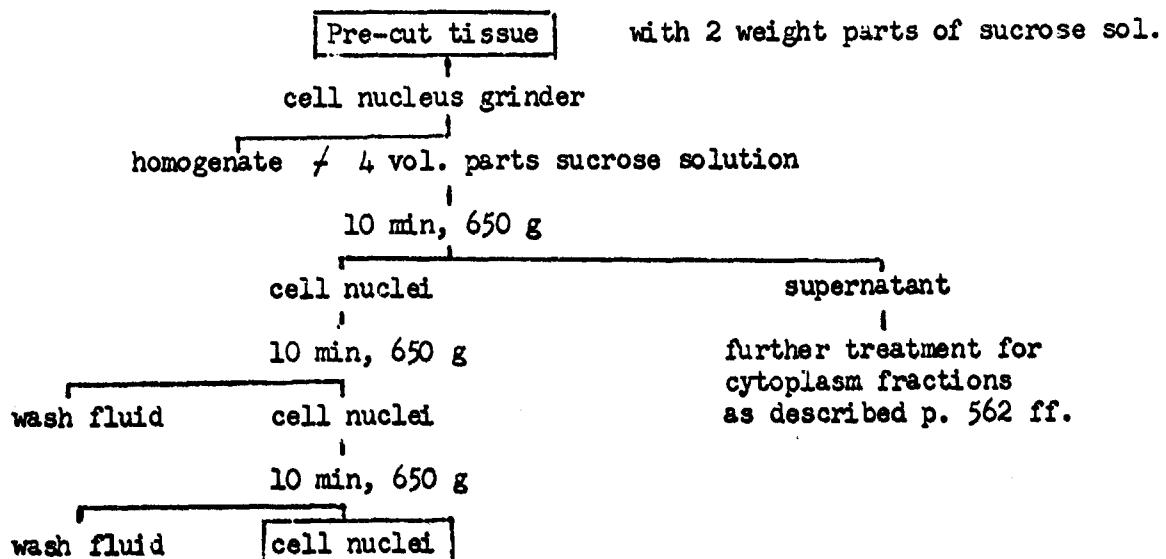
The cylinder is surrounded by a cooling mantle filled with an ice-sodium chloride mixture; the receptacle under the cylinder is also cooled with ice. Modifications of this grinder concern the arrangement of the motor, which may also be attached beneath the grinder proper; in the case of a stationary piston the cylinder, surrounded by the cooling mantle, is raised or lowered in order to adjust the gap; the shaft of the piston requires a catcher that leads the homogenate leaving the cylinder to the receiving vessel. The rotary current motors with 1 HP capacity have selective rates of 700 and 1,400 RPM.

The pre-cut tissue placed in the funnel of the nucleus grinder retains temperatures below 4°C , even after operation for hours, if the temperature of the cooling mantle is between -8 and -12°C . The dilution of the tissue with 30% sucrose solution, including the amounts used for the rinsing of the glass homogenizer and the filter cloth, measures 2 vol. parts sucrose solution to 1 weight part of tissue. The adjustment of the gap initially is made manually with the motor stopped, then at low rates of speed; after some experience the gap required for proper grinding is recognized by the number of drops flowing off on the bottom; depending on the type of tissue and animal species, the flux reaches 0.5-5 cc/min. at 1,400 RPM.

Of the total tissue suspension placed in the grinder, the last third or fourth is discarded by discontinuation of the grinding process after the passage of 65-75% of the volume. It has been demonstrated that the largest particles of the tissue suspension remain in the entry funnel for the longest time; if the homogenization is continued to its termination, tissue membranes and other cellular debris will ultimately be passed through and will thereby decrease the purity of the cell nuclei, since they will settle out with the latter during centrifugation.

For the purpose of differential centrifugation the ground tissue homogenate is diluted with 4 col. 30% sucrose solution and then spun at 650 g for 10 minutes. The supernatant fluid is drawn off and may serve for the production of mitochondria, microsomes and cytoplasma, as described on p. 562 ff. The cell-nuclear sediment is resuspended by means of a glass rod in one-half the original volume of sucrose solution and again centrifuged for 10 minutes at 650 g. After the wash liquid is discarded, another identical separation is made with one-half volume 30% sucrose solution ($= \frac{1}{4}$ of the starting volume). The resulting sediment consists practically only of pure cell nuclei.

The following chart depicts the principle of the operation.



If, for various reasons, the sucrose is to be removed, this requires a 4-fold wash in 2 vol. distilled water or vigorous dialysis against distilled water for several hours, until only traces of sucrose are present. Freeze drying is suitable for the preservation of cell nuclei; the dry powder then contains 60-80% sucrose. The production of acetone dry powder may be coupled with the removal of sucrose; the isolated cell nuclei are twice washed with 2.5 vol. acetone, containing 20% water, and then twice with pure acetone. The sucrose content of the cell nuclei is reduced to imperceptibility thereby. The initial admixture of water to acetone is necessitated by the limited solubility of sucrose in acetone. The same principle permits, for instance, the production of an acetone dry powder from mitochondria (cf. also p. 581 f.) For a convenient method of sucrose analysis see (4).

For the evaluation of the yield it may be considered that, in most animal tissues, 8-12% of the volume or the nitrogen content, respectively, is taken up by the cell nuclei. The necessary numerical values are obtained by the measurement of the homogenate volume and the determination of N in the washed sediment. The percentual expression of purity refers to the number of impure cell nuclei (or cell-nuclear debris) or intact cells among 400 particles of the cell nuclear sediment to be counted under the microscope. Usually, a purity of 97-99% is obtained for cell nucleus preparations. Fig. 10 shows cell nuclei prepared according to Lang and Siebert. The cell nucleus of every individual tissue has a characteristic appearance, so that frequently the organ from which the cell nuclei are obtained may be identified during the scanning of a cell-nuclear smear.

For the morphological determination of purity, a small part of the sediment, thoroughly stirred with a glass rod, is smeared on a slide and stained after May-Gruenwald immediately after drying at the air. In most cases it is recommended to cover the smear without additional fixation for 3-5 minutes with the staining agent and then to dilute the stain to one-half by the addition of distilled water with a pipette. After 10 minutes the smear is thoroughly rinsed with distilled water. After air-drying, the smears are examined without oil immersion at about 200X magnification. The sucrose on the slide usually absorbs some of the stain; adjustment of the micrometer knob will allow the immediate differentiation of sucrose crystals from possible cellular particles. A "bedding" with glycerol-gelatine is frequently advantageous.

The essential criteria for evaluation are:

1. Absence of cytoplasmic elements (cytoplasmic margin, reddish stain, granulated; mitochondria; membranes and other cellular debris).
2. Good staining properties of the cell-nuclear contents, usually appearing to be homogeneous, nucleoli distinguish themselves by a deeper blue stain.
3. Round or spindle-shaped form with smooth contours.

A special kind of glass homogenizer (5), suitable only for manual operation, has been described by Lang and Siebert (6). It is routinely used for the preliminary diminution of the tissue before it is placed in the grinder (see p. 569). However, it is also suited to the production of a cell nucleus fraction of 90-95% purity (the essential effect of the cell nucleus grinder consists, according to present experience, in the improved purity of the cell nuclei, which is often decisive in experiments). The operation is depicted in Fig. 11. The piston is moved up and down manually. During the downward thrust the glass needle valve is closed; for this reason all tissue elements are pressed up between the piston and the tube's wall. Upon upward displacement, the valve opens and a fresh supply of tissue suspension reaches the space compressed by the piston in its next downward motion, without the danger of losses due to ejection. It is recommended to use several pistons with different diameters; first the loosest is inserted and last the tightest. After frequent repetition of the motion, the piston fails to meet resistance. Tissue homogenates examined in this condition yield 90-95% pure cell nuclei, if they are pressed through a filter cloth and centrifuged, as described above. Studies of yield and purity (7) have shown that the yield is 50-100% higher than by use of the cell grinder. The saving in time is 2-4-fold. The procedure can be applied to swine kidney and swine pancreas, as well as to rat liver, but not to human placenta (the cell grinder has the same practical result) or bull prostate (due to the consistency of the tissue). If absolutely pure cell nuclei are to be used, the cell nucleus grinder is indispensable.

A homogenate produced in this manner is eminently suitable for the counting of cell nuclei (cf. also p. 573) and therefore indirectly for the cell count of a given quantity of tissue.

An additional instrument (Fig. 1, p. 562) facilitates the drawing off of one or more layers that occur in the centrifugal tube after spinning. The procedure is readily apparent. The drawn-off liquid or sedimental layer collects in vessel B and may easily be removed from it. Suction is applied through a thin hose by mouth or from the water stream pump.

For the biochemical analysis of the purity of cell nuclei, the complete absence of oxydation ferments (like the succinoxidase test), a comparison of the DNA content with the starting material (also suitable for the determination of yield), or the determination of the ratio of DNA to RNA, the value of which in cell nuclei from animal tissue generally amounts to 5:1-30:1 (8), may be used. For additional normal values, see Table 3, p. 556.

The counting of cell nuclei after Price, Miller, Miller and Weber (9). An homogenate of rat liver or other organs (see p. 542 f. and 562 ff.), produced in a suitable manner, is diluted with 0.88 m sucrose solution, if necessary, until about 50 cell nuclei are contained in 1 cmm during the final count. Usually, the 2-3-fold sucrose volume of the original homogenate is necessary. Subsequently this diluted homogenate is further diluted 1:20 with 3% acetic acid, containing 0.02% methyl green, and

counted in an erythrocyte counting chamber in the usual manner.

According to Laird (10) the homogenate produced in 0.88 m sucrose solution is diluted 1:40 with 3% acetic acid, containing 1:10,000 gentian violet in dissolved form. Mizen and Petermann (11), who worked with mouse spleen, use 0.83 m sucrose solution with 0.0018 m CaCl_2 for the production of the homogenate and 6% acetic acid with 0.0018 m CaCl_2 for dilution. In the case of leukemic spleens the Ca concentration must be raised to 0.0023 m.

NOTES

(1) Lang, K. and G. Siebert: B.Z. 322, 360 (1952). --- (2) For example, "WWb" and "P 4452" of the firm Beth, Machine Factory, Luebeck, or "1400" of the firm Ude, K.G., Kallefeld via Kreiensen. --- (3) Manufacturer: B. Buehler, Instrument Factory, Tuebingen. --- (4) Siebert, G., K. Traenckner and K. Lang: Naturwiss. 41, 460 (1954). --- (5) Available from B. Braun, Instrument Plant, Melsungen. --- (6) Lang, K. and G. Siebert: B.Z. 322, 360 (1952). --- (7) Siebert, G., K. Lang, S. Lang and D. Neumann: Not published. --- (8) Lang, K. and G. Siebert: Die chemischen Leistungen der morphologischen Zellelemente. In Flaschentraeger-Lehnartz Vol. 2/1, p. 1064 ff. --- (9) Price, J.M., E.C. Miller, J.A. Miller and G.M. Weber: Cancer Res. 10, 18 (1950). --- (10) Laird, A.K.: Exp. Cell Res. 6, 30 (1954). --- (11) Mizen, N.A. and M.L. Petermann: Cancer Res. 12, 727 (1952).